# Influence of Lipid Bilayer Composition on Plasma Membrane Repair Capacity

Undergraduate Research Thesis

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by

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**Abstract**

Lipids are a major constituent of the plasma membrane where they are found in great diversity of structure and proportions. The sarcolemmal membrane also exhibits this diversity which affects its fluidity and integrity. Modifying dietary fat intake has been shown to be an effective method for changing the fatty acid (FA) composition of the membrane. Membrane FA composition influences the fluidity and diffusion process of the membrane, which may serve as a possible avenue for managing muscular dystrophies. In addition, varying membrane composition may influence various protein activities by affecting the recruitment of lipid-binding proteins and protein homeostasis. Thus, became the hypothesis that altering the plasma membrane's FA composition will have an effect on the resealing process of the plasma membrane. To test this hypothesis, laser damage assay was used on flexor digitorum brevis (FDB) muscle cells from sedentary wild type and dysferlin knockout mice fed diets high in oleic acid or palmitic acid for a period of 5-6 weeks. A multiphoton laser was used to injure the muscle cells in presence of the lipophilic dye FM4-64. The resealing capacity was measured through observing the change in dye fluorescence over time. The laser injury assay was a measure of the repair kinetics of the plasma membrane. To test the integrity of the sarcolemma and muscle damage, IgG staining was done using 488 goat anti mouse antibody on histological sections of the extensor digitorum longus (EDL) muscle from wild type and dysferlin knockout mice that were fed diets rich in palmitic acid or oleic acid. The results did not show a significant difference in repair kinetics or integrity of the sarcolemma in mice that were fed diets high in oleic acid versus those that were fed diets high in palmitic acid. These were unexpected results that need further investigation starting with mass spectrometry assays to confirm changes in the membrane composition.

## Introduction

Maintenance of plasma membrane integrity is essential for healthy cell function and disruption of membrane integrity contributes to progression of myopathies (4). In striated muscle cells a healthy membrane, or sarcolemma, is not only achieved by avoiding disruptions and injury but also by having an efficient functional repair system that reseals gaps in membrane post disruption. Repair is often done through patch formation during which $Ca^{2+}$ dependent exocytosis vesicular trafficking brings vesicles to the wound site to close the gap (6). Interactions among membrane proteins and lipids heavily influences cellular integrity and altering these interactions may result in dramatic changes. Dysferlin is a transmembrane protein that is essential for efficient membrane repair through the patch formation mechanism. The $Ca^{2+}$ dependent dysferlin protein helps intracellular vesicles to fuse together and with the membrane (17) through exocytosis to form a patch that will repair injuries in the sarcolemma. Mutations in the dysferlin gene result in a non-functional protein and could lead to limb-girdle muscular dystrophy type 2B. Dysferlin-deficient mice are a mouse model of limb-girdle muscular dystrophy and they were used for this project. Other proteins that are important for the membrane repair process include mitsugumin 53 (MG53) and dystrophin. MG53 is another vital protein that is associated in the patch formation membrane repair mechanism (2). MG53 proteins are tethered to the intracellular vesicles taking part in the fusion of the vesicles to form the membrane patch.

Membrane integrity is compromised in muscular dystrophies and so is the repair process, but not much is known about the influence of different fatty acids on the skeletal muscle membrane. Phospholipids are the major component of plasma membrane and each phospholipid has two fatty acid tails, so fatty acids are important for membrane structure and characterization. Saturated fatty acids, such as palmitic acid, lack double bonds in their structure, which makes it possible for them to pack closely together. The close packing of unsaturated fatty acids in the plasma membrane add rigidity to the fluid and dynamic structure of the membrane. Sarcolemmal membrane FA composition is different in wild type and dystrophic muscle sections and it has been proven in literature that variation to membrane composition can be

induced through dietary changes (1,5,7,9,11,12). Striated muscle samples from control and DMD patients showed an abundance of monounsaturated fatty acids in the most damaged sections of the dystrophic muscle sections (14,15). Changes to FA sacrolemmal membrane composition has the potential to alter the fluidity and thus integrity and repair kinetics of the plasma membrane and could be used to mange the progression of muscular dystrophies.

Plasma membranes are composed of a multitude of fatty acids (FA) with varying properties ultimately affecting the permeability and rigidity of the membrane. The FA composition of different diets influences the lipid composition of skeletal muscle (1). Membrane fluidity was significantly reduced in presence of the saturated FA palmitate in comparison to the polyunsaturated FA linoleate (8). The fluidity of membrane may influence the repair capacity or integrity of membrane by affecting rates of molecule diffusion across the membrane and fusion of intracellular vesicles together and with the plasma membrane. Post chemical or physical disruption, the plasma membrane thermodynamically reseals either through mechanical tension from integral proteins, recruitment of intracellular vesicles to form a repair patch, shedding of injured membrane, or endocytotic mechanisms (2). Smaller disruptions to the plasma membrane tend to be repaired through mechanical tension, budding, or exocytosis (2). FA composition of membrane has a modulating effect on protein activities and gene expression (5). Thus, for smaller disruptions, it is possible that influencing the integrity of membrane bilayer through changes to the lipid composition may enhance the repair process. The ability to change membrane composition through manageable dietary changes holds the potential of improving muscle integrity in patients suffering of muscular dystrophy.

A healthy and resilient sarcolemma is essential for controlling the proregression of muscular dystrophies. As stated earlier, FA composition of skeletal muscle sarcolemma has been shown to be changed through dietary changes; diets high in the monounsaturated FA, oleic acid, such as the Mediterranean diet reflect an increase of oleic acid in plasma membrane of human and rat cells and increase membrane fluidity (1,5,7). Dystrophic sarcolemma shows an increase in total FAs compared to sarcolemma from wild type

mice (9) and specifically an increase in oleic acid levels (11,12). Diet rich in polyunsaturated fatty acid (a-linolenic acid) made mdx muscle more susceptible to sarcolemmal leakiness (10). However, a different study found that diet high in a-linolenic acid prevented dystrophic degeneration of muscle morphology and function (13). Imposing cellular changes through dietary changes is an easy and powerful therapeutic approach. While dietary changes cannot be used as the sole treatment of muscular dystrophies, they may be an effective way of managing the progression of muscular atrophy disorders in conjunction with other treatment options.

The purpose of this study was to evaluate the relationship between fatty acid composition of sacrolemmal membrane and the integrity and repair kinetics of the sarcolemma. Alterations in FA levels of the sarcolemma have been documented previously; this study looks for the effects of changing the membrane composition on its integrity specifically by increasing oleic acid (monounsaturated fatty acid) or palmitic acid (saturated fatty acid) levels in the sarcolemma of both wild type and dysfeerlin-deficient mice. Membrane repair kinetics were measured through laser injury assay and sarcolemma integrity was measured through immunoglobin-G staining. No significant changes were observed by increasing levels of oleic acid or palmitic acid. Increasing the levels of oleic acid in the sarcolemma was expected to add fluidity to the membrane and enhance the fusion of intracellular vesicles with the sarcolemma. This increased fluidity and enhancement of fusion was expected to result in more efficient repair kinetics and higher plasma membrane integrity, which was not observed. Increasing palmitic acid levels in the sarcolemma was expected to add rigidity to the sarcolemma thus decreasing the efficiency of vesicular fusion and thus plasma membrane resealing kinetics. Again, this reduction in efficiency of resealing kinetics was not observed. Therefore, further experiments are needed to investigate these unexpected results.

## Results

Mixed sex group of wild type mice were fed diets high in oleic acid or palmitic acid for a period of 5-6 weeks. After the feeding period was over, the flexor digitorum brevis (FDB) muscles from the sedentary mice were collected following standard surgical procedure for laser injury assay. A multiphoton infrared laser was used to injure FDB muscles collected to measure changes in membrane resealing kinetics. The muscles were placed in a glass bottom MatTek dish with 2µm FM4-64 dye solution. The infrared laser was directed at the membrane to induce an injury in the sarcolemma causing the lipophilic FM4-64 dye to enter injury site and attach to the exposed lipids of membrane. More efficient membrane resealing equates to less dye influx.

From the same group of wildtype mice, extensor digitorum longus (EDL) muscles were collected to look for changes in membrane integrity through mouse immunoglobin G (IgG) staining. EDL histological sections were stained with fluorescent-labeled antibodies against mouse IgG. If IgG is detected, it indicates that the membrane was disrupted and then resealed. Detection of high levels of IgG indicates that the membrane was more fragile, and thus more repair was needed.

**Increased palmitic acid or oleic acid levels in the sarcolemma of healthy mice do not change membrane repair kinetics.**

Wild type mice fed diets high in oleic acid or palmitic acid showed no significant changes in sarcolemmal membrane resealing kinetics. Figure 1 shows representative images from laser injury assay on the FDB of wildtype mice. Figure 1A shows images of the FDB of wildtype mice fed diets high in palmitic acid both before and after injury using the infrared laser. Figure 1B shows images of the FDB of wildtype mice fed diets high in oleic acid both before and after injury using the infrared laser. In figures 1A and 1B the arrows point the injury site before and after injury. After injury the red areas pointed to with the arrows show the FM4-64 dye influx to the injury site. Quantification of the laser injury data is preseented in figure 2. Figure 2A shows changes in fluorescence over time. The first three data points on 2A graph are collected before injury is induced. FDB from both palmitic and oleic acid diet groups display the same typical pattern of membrane repair. A large influx of FM4-64 dye, measured as fluorescence, at the

instance of injury is followed by a plateau of florescence changes indicating that the membrane is repaired. Figure 2A shows no significant difference in the sarcolemma resealing kinetics between wildtype mice fed diets high in oleic acid or palmitic acid. The area under the curve (AUC) graph (figure 2B) shows that the amount of dye influx was not significantly different between the oleic acid and palmitic acid groups either. Thus, membrane repair kinetics are not influenced by increasing palmitic or oleic acid levels in membrane because rate of florescence change and amount of dye influx were comparable in sedentary wild type mice on diets high in oleic or palmitic acid.

**Increased palmitic acid or oleic acid levels in the sarcolemma of healthy mice do not alter membrane integrity.**

Wild type mice fed diets high in oleic acid or palmitic acid showed no significant changes in sarcolemmal membrane integrity. Figure 3 shows representative images of IgG stained EDL muscles of wildtype mice. Figure 3A shows a representative image of IgG stained section from EDL of a wildtype mouse fed a diet high in oleic acid. Figure 3B shows a representative image of IgG stained section from EDL of a wildtype mouse fed a diet high in palmitic acid. To compare images from the two groups of mice, the percent area of positive fibers was measured using ImageJ software. There is not a significant difference in the percent area of positive fibers measured in EDL from mice fed diets rich in oleic acid or palmitic acid. These observations were confirmed by quantification of the percent area of positive fibers in figure 4. The percent area of positive fibers was not significantly different between the two groups of wildtype mice. Thus membrane integrity was not affected by oleic acid or palmitic acid diets because the percent area of IgG-positive fibers is comparable in the two groups of wildtype mice.

Data from wild type mice did not show a significant difference in membrane repair kinetics or membrane integrity by increasing palmitic acid or oleic acid levels in the plasma membrane. Sedentary wild type mice muscles experience regular daily stress; however, that may have not been enough to show the effects of varying membrane lipid composition. Possibly because the healthy mice have an efficient $Ca^{2+}$ dependent membrane repair mechanism, varying the levels of FA in the plasma membrane is not enough to show their effect on membrane repair or integrity.

The dysferlin protein is associated in sarcolemmal membrane repair. Dysferlin knockout mice model muscular dystrophy, specifically limb-gridle muscular dystorphy. Dysferlin-deficient mice have compromised muscle fibers because of their lack of a functional dysferlin protein. Effects of varying membrane lipid composition were assessed in dysferlin-deficient mice again through laser injury assay and IgG staining to look for changes in membrane repair kinetics or integrity.

**Increased palmitic acid or oleic acid levels in the sarcolemma of dystrophic mice do not change membrane repair kinetics.**

Dysferlin-Deficient mice fed diets high in oleic acid or palmitic acid showed no significant changes in sarcolemmal membrane resealing kinetics. Even with the compromised muscles in dysferlin-deficient mice, membrane repair kinetics did not significantly vary between the palmitic acid and oleic acid diet groups. Figure 5 shows representative images of laser injury of EDL muscles of dysferlin-deficient mice. Figure 5A and 5B show images before and after laser injury of EDL muscles of dystrophic mice fed diets high in palmitic acid and oleic acid, respectively. The change in florescence over time was comparable in both groups of dystorphic mice (figure 6A). The AUC graph shows that the total amount of dye influx is also comparable in the different diet groups of dystrophic mice (figure 6B). The total dye influx is greater in the dysferlin-deficient mice compared to the wild type mice because dysferlin is essential for membrane repair. So, without a functional dysferlin protein, the muscles of dystrophic mice are not able to repair disruptions to the membrane efficiently. However, the data shows no significant difference in the resealing kinetics between the dystrophic mice groups, thus increased palmitic acid or oleic acid levels in the sarcolemma of dystrophic mice do not alter membrane repair kinetics.

**Dysferlin-Deficient mice fed diets high in oleic acid or palmitic acid showed no significant changes in sarcolemmal membrane integrity.**

Dysferlin-Deficient mice fed diets high in oleic acid or palmitic acid showed no significant changes in sarcolemmal membrane integrity. Figure 7 shows representative images of IgG stained histological EDL sections from dysferline-deficient mice. The percent area of IgG-positive fibers is comparable in both palmitic acid and oleic acid groups of dysferlin-deficient mice. Figure 7A shows representative image of

IgG stained EDL sections of dystophic mice fed diets high in oleic acid. And figure 7B shows representative image of the dystrophic mice fed diets high in palmitic acid. The overall percent area of IgG-positive fibers in dysferlin-deficient mice is greater than in wild type mice due to dysferlin's role in membrane repair. However, within the dystrophic mice group, comparing the mice fed diets rich in oleic acid vs palmitic acid, there is no significant difference in the percent area of positive fibers as presented in figure 8. Daily stress on muscles of dysferlin-deficient mice is not repaired as efficiently as in the wild type mice, which explains the significant difference in the percent area of positive fibers between wildtype and dysferlin-deficient mice. Thus, increasing palmitic acid or oleic acid levels in the sarcolemma of dystophic mice did not play a role in modifying the integrity of the plasma membrane.

## Discussion

The goal of this project was to investigate whether changing fatty acid composition of plasma membrane affects the integrity or repair kinetics of sarcolemmal membrane. Results suggest that increasing oleic acid or palmitic acid concentration in the membrane does not result in significant changes to membrane integrity or repair kinetics. However, this project is a preliminary investigation of the effects of varying lipid composition in membrane. There is a large variety of fatty acids that may be manipulated to test for effects in sarcolemma and further research is needed to fully evaluate the influence of varying lipid composition on membrane integrity and repair capacity. For example, cholesterol is found in the membrane and it's responsible for adding rigidity to the membrane. Increasing cholesterol levels in the membrane or stripping the membrane of cholesterol may show a significant change to the membrane integrity or resealing kinetics. However, the first follow-up step after the unexpected results of this project is to do mass spectrometry assay on frozen muscle samples from the mice used in this project. Mass spectrometry can be used to indentify the chemical structure of the plasma membrane and show whether the sarcolemma composition was changed in the mice fed the diets rich palmitic acid or oleic acid. While it has been proven that feeding mice diets rich in specific fatty acid for a period of 6 weeks is sufficient to enrich the plasma membrane with the respective fatty acid, it is important to confirm that this was the case in this study. Because the results showed no significant differences between the oleic acid and palmitic acid groups, we must confirm that the plasma membrane composition was changed in order to fully establish that increased palmitic acid or oleic acid levels in the sarcolemma do not change membrane repair kinetics or integrity.

Wild type sedentary mice experience minimal muscle damage, which may have not been sufficient to show changes in membrane integrity and repair. Dysferlin-deficient mice were used to model compromised muscle tissue as would be the case in patients of muscular dystrophies. In both strains of mice used in the study, injury to the membrane was through laser injury which is typically a localized disruption. Other methods such as downhill running could be used to induce more stress and injury to the muscles that may require specific fatty acid composition for efficient repair. Larger-scale injuries to the

membrane were not tested in this study. It is possible that repair kinetics of larger injuries have a different response to membrane changes in fatty acid composition due to the different repair mechanisms. Supplementing cells with certain lipids allows the cells to implement these lipids in the membrane changing its composition (3) which makes it possible to study the influence of varying lipid composition in vitro. So changing the composition of other lipids and components of the membrane is another potential future study.

Increased oleic acid levels in dystrophic muscles indicates that oleic acid is important for maintaining a healthy sarcolemma. The cytoskeletal network adds tension to the membrane which needs to be elevated to a certain degree for successful membrane resealing (16). Changing the lipid composition of the sarcolemma changes the properties of the membrane so even though the results of this study did not show any significant changes, further research is needed to confirm the role of fatty acids in maintaining membrane integrity and how it can be used to control the progression of muscular dystrophies.

## Figures

**Figure 1:** Representative images of laser injury on FDB of wildtype mice

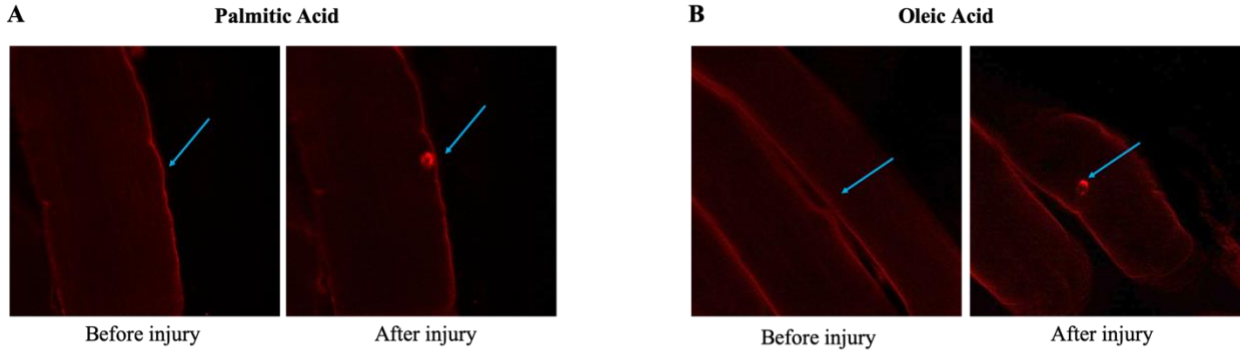


**Figure 1:** Wild type mice fed diets high in oleic acid or palmitic acid showed no significant changes in sarcolemmal membrane resealing kinetics. **1A & 1B:** representative images of laser injury on EDL of wildtype mice fed diet rich in palmitic acid and oleic acid, respectively. Arrows point to injury site before and after injury. The red injury site on the muscle fibers after injury shows the FM4-64 dye influx to the injured sarcolemma.

**Figure 2:** Laser Injury on FDB of wildtype mice

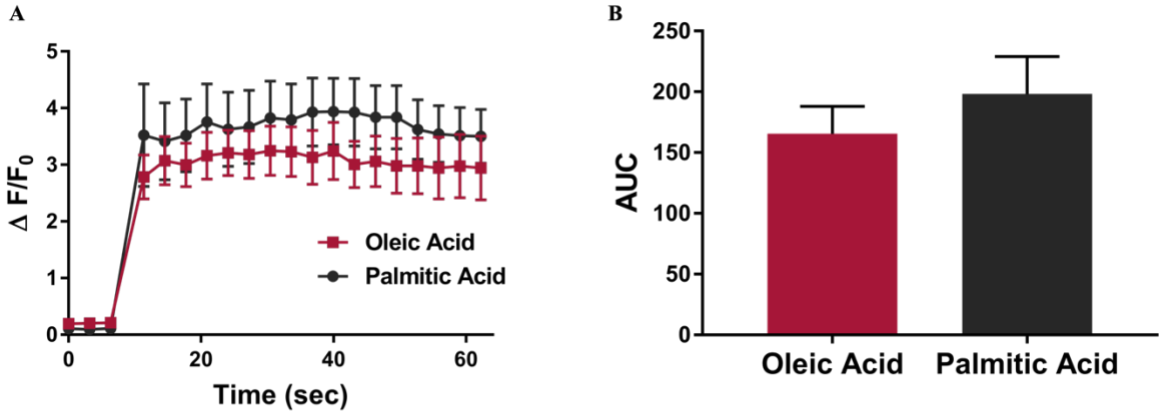


**Figure 2:** Increased palmitic acid or oleic acid levels in the sarcolemma of healthy mice do not change membrane repair kinetics. **2A:** change in fluorescence over time graph shows no significant difference in the repair kinetics between the oleic acid diet group and the palmitic acid diet group. **2B:** area under the curve graph shows no significant difference in the amount of FM4-64 dye influx to injury site between the oleic acid diet group and the palmitic acid diet group.

**Figure 3:** Representative images of IgG stained EDL of wildtype mice

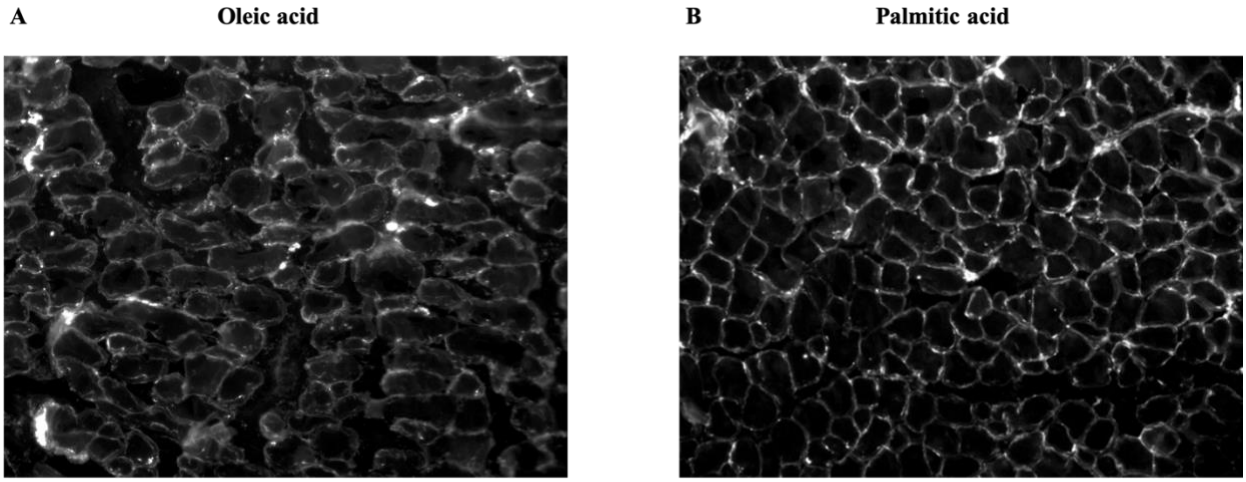


**Figure 3:** Wild type mice fed diets high in oleic acid or palmitic acid showed no significant changes in sarcolemmal membrane integrity. **3A & 3B:** IgG stained EDL of wildtype mice fed diets rich in oleic acid and palmitic acid, respectively.

**Figure 4:** Quantification of IgG staining of EDL of dysferlin-deficient mice

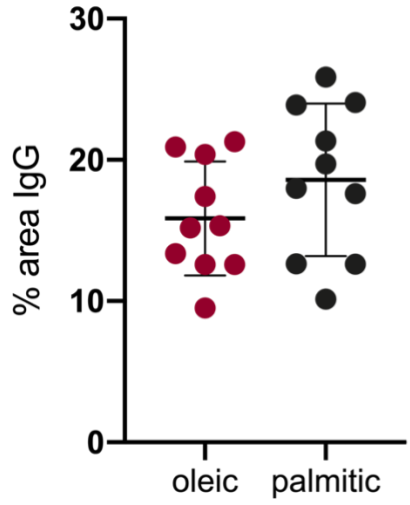


**Figure 4:** Increased palmitic acid or oleic acid levels in the sarcolemma of healthy mice do not alter membrane integrity. Quantification of percent positive area of IgG stained EDL muscles of wildtype mice showed no significant difference between the mice fed diets high in oleic acid and palmitic acid.

**Figure 5:** Representative images of laser injury on FDB of dysferlin-deficient mice

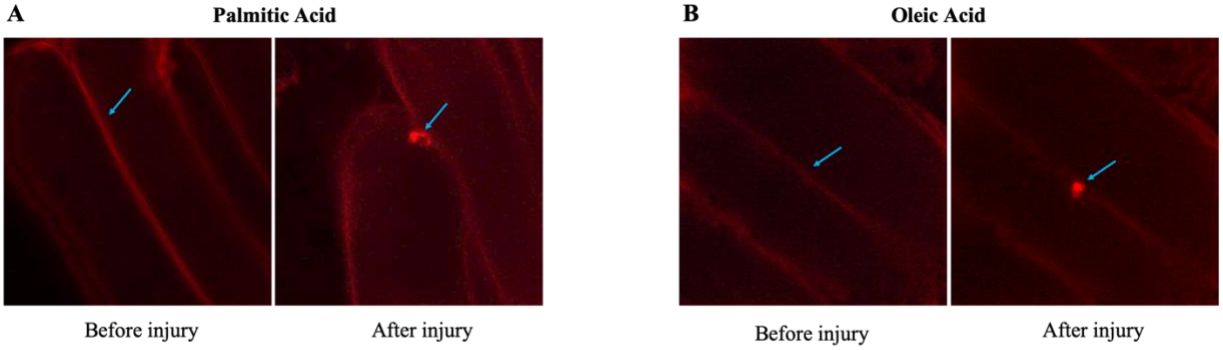


**Figure 5:** Dysferlin-deficient mice fed diets high in oleic acid or palmitic acid showed no significant changes in sarcolemmal membrane resealing kinetics. **5A & 5B:** representative images of laser injury on EDL of dysferlin-deficient mice fed diet rich in palmitic acid and oleic acid, respectively. Arrows point to injury site before and after injury. The red injury site on the muscle fibers after injury shows the FM4-64 dye influx to the injured sarcolemma.

**Figure 6:** Laser Injury on FDB of dysferlin-deficient mice

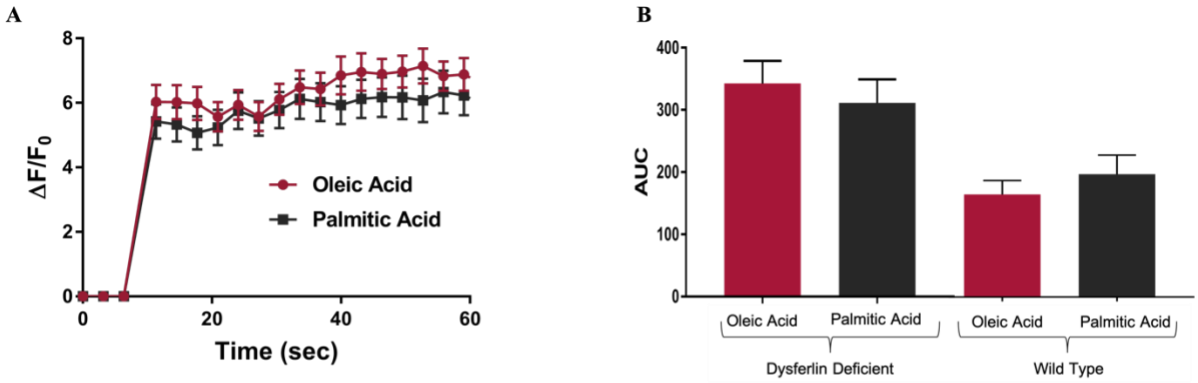


**Figure 6:** Increased palmitic acid or oleic acid levels in the sarcolemma of dysferlin-deficient mice do not change membrane repair kinetics. **2A:** change in fluorescence over time graph shows no significant difference in the repair kinetics between the oleic acid diet group and the palmitic acid diet group. **2B:** area under the curve graph shows no significant difference in the amount of FM4-64 dye influx to injury site between the oleic acid diet group and the palmitic acid diet group. Comparing dysferlin deficient group to wild-type group shows that more FM4-64 dye rushes to injury site in the dysferlin deficient group compared to the wild-type group, which is expected because dysferlin deficient mice don't have an efficient membrane repair mechanism since they lack dysferlin protein.

**Figure 7:** Representative images of IgG stained EDL of dysferlin-deficient mice

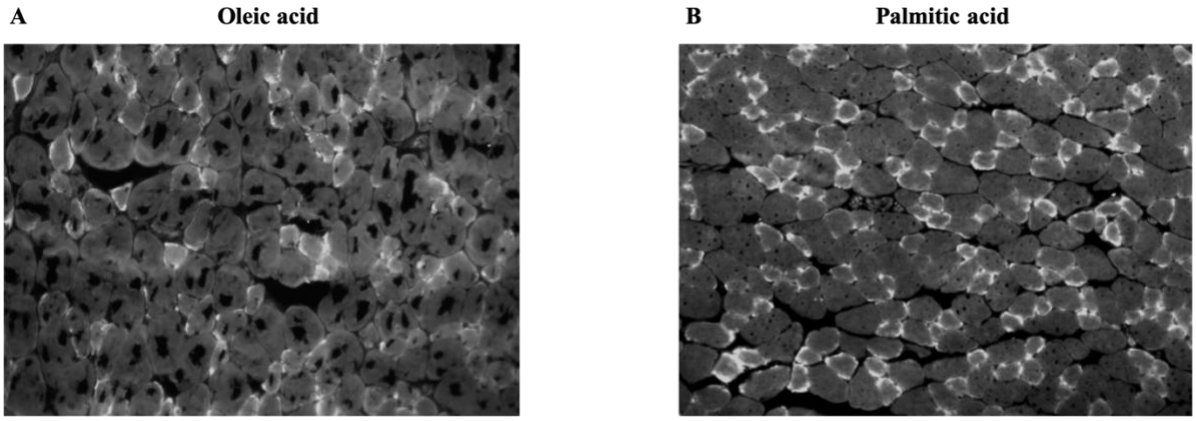


**Figure 7:** Dysferlin-deficient mice fed diets high in oleic acid or palmitic acid showed no significant changes in sarcolemmal membrane integrity. **7A & 7B:** IgG stained EDL of wildtype mice fed diets rich in oleic acid and palmitic acid, respectively.

**Figure 8:** Quantification of IgG staining of EDL of dysferlin-deficient mice

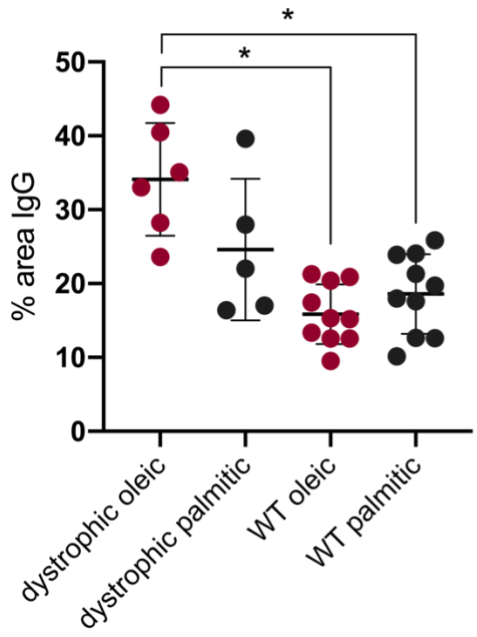


**Figure 8:** Increased palmitic acid or oleic acid levels in the sarcolemma of dysferlin-deficient mice do not alter membrane integrity. Quantification of percent positive area of IgG stained EDL muscles of dysferlin-deficient mice showed no significant difference between the mice fed diets high in oleic acid and palmitic acid. The percent area of positive fibers is greater for the dystrophic mice compered to wild type, which is expected because the dystrophic mice do not have a function dysferlin protein.

## Methods

**Measuring membrane repair kinetics through laser injury.** FDB muscles were isolated from wild-type and dysferlin-deficient mice using a standard surgical procedure. The isolated muscles were placed in a 35 mm MatTek glass bottom dish with 2mM Tyrode's solution. Membrane damage was induced using infrared laser (23% power) of the Olympus FV1000 multi-photon laser microscope in presence of 2µM FM4-64 dye. The FM4-64 dye is a lipophilic dye and measurement of its entry into the muscle fibers was recorded for 100 seconds at 3 second intervals to evaluate membrane repair kinetics. ImageJ was used to analyze the laser injury data. Intensity of fluorescence at the injury site and its change over time correlates with the repair kinetics of the sarcolemma.

**Analyzing muscle damage and membrane integrity through immunoglobin G (IgG) staining.** IgG is the most abundant antibody produced by the body, so high levels of IgG in muscle sections indicate muscle damage and compromised membrane integrity. EDL muscles were isolated from wild-type and dysferlin-deficient mice using standard surgical procedure. The EDL muscles were embedded in optimal cutting temperature compound (OCT) and frozen using flash freezing techinique in liquid nitrogen to avoid formation of large ice cyrstals and damaging the samples which were then stored in -80ºC freezer. A Leica CM1510S cryostat was used to cut the frozen samples into thin sections that were then placed on slides. The sections were blocked with 2.5% BSA in PBS solution for 1 hour and then stained with 0.01% 488 goat anti-mouse IgG solution in 2.5% BSA for 2.5 hours at room temperature, washed in PBA, and mounted with coverslips and stored in 4ºC fridge. Images of the stained sections were taken using an Olympus FluoView FV1000 confocal microscope. The images were analyzed using ImageJ to obtain percent area of positive sections for each muscle section.

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